

AD_____

Award Number: DAMD17-97-1-7250

TITLE: Structural Basis of EGFR Dimerization for Drug Design

PRINCIPAL INVESTIGATOR: James Marks, M.D., Ph.D.

CONTRACTING ORGANIZATION: The Regents of the University
of California
San Francisco, California 94143-0962

REPORT DATE: September 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 2000	3. REPORT TYPE AND DATES COVERED Final (01 Sep 97 - 31 Aug 00)		
4. TITLE AND SUBTITLE Structural Basis of EGFR Dimerization for Drug Design		5. FUNDING NUMBERS DAMD17-97-1-7250		
6. AUTHOR(S) James Marks, M.D., Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Regents of the University of California San Francisco, California 94143-0962 E-Mail: marksj@anesthesia.ucsf.edu		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER		
11. SUPPLEMENTARY NOTES Report contains color.		20011127 047		
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) Breast cancer occurs when there is a transition from normal breast epithelial cell behavior to that of uncontrolled cell growth. Cell surface receptors and specific growth factors play a crucial role in this transition. For this project, we proposed to evaluate the role of the epidermal growth factor receptor (EGFR) in breast cancer by expressing and purifying EGFR and solving the atomic structure. Antibodies generated from phage libraries would be used to: 1) facilitate structure solution, and 2) deliver drugs to EGFR expressing cells. EGFR was expressed at high levels and purified to homogeneity, but diffraction quality crystals were never obtained. As a result, efforts focused on antibody generation. A large panel (33) of human scFv antibodies to EGFR were isolated by selection on purified recombinant EGFR or on cells expressing EGFR. For cell selections, a method was developed which allowed direct selection from phage libraries of antibodies which trigger receptor mediated endocytosis. We show that this approach can be used either on tumor cell lines which overexpress the receptor or on cells transfected with the EGFR gene. This permits use of this methodology on transfected cells and provides a means of making antibodies without the need for protein expression and purification. We show that internalizing EGFR antibodies can be used to deliver cytotoxic agents into the cytosol of EGFR expressing tumor cells by construction of immunoliposomes bearing an EGFR antibody on their surface. We are in the process of constructing and evaluating such an agent for breast cancer therapy.				
14. SUBJECT TERMS Breast Cancer, immunotherapy, epidermal growth factor receptor, phage antibodies			15. NUMBER OF PAGES 27	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

MA Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

MA In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

MA In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

MA In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

jo mmb
PI - Signature

August 14, 2001
Date

Table of Contents

Section	Page numbers
1. Front Cover	1
2. Standard Form 298	2
3. Foreword	3
4. Table of Contents	4
5. Introduction	5-7
6. Body of Report	7-9
7. Key Research Accomplishments	9
8. Reportable Outcomes	9
9. Conclusions	9
10. Personnel Supported by the Grant	9
11. References	10-12
12. Appendices (Papers published)	13-27
Appendix 1: Heitner et. al. J. Immunol. Meth., 2001	

5. Introduction

Systemic therapy of human malignancies depends upon differential toxic effects on malignant and normal cells. Unfortunately, traditional cancer therapeutic agents, including chemotherapy and radiation treatment, cannot distinguish between normal cells and tumor cells and hence damage and kill normal proliferating tissues. Therefore a major goal of cancer research has been development of drugs to specifically kill tumor cells. To achieve this goal, researchers have attempted to identify cell surface markers on tumors that are qualitatively or quantitatively different from normal cells. These tumor markers could provide the needed specificity for cancer therapy and form the basis of diagnostic and prognostic tests. Tumor specific markers include B-cell idiotypes (1, 2) and components of epithelial core mucins (3). Quantitatively different tumor associated markers include epidermal (EGFR) and vascular endothelial cell growth factor receptors (VEGFR) (4-7).

5.1. Role of growth factor receptors in normal and aberrant cell growth

Receptors located in the cell membrane permit communication between cells and thus are arguably the most important molecules in the signal transduction pathway. Receptors with intrinsic tyrosine kinase activity mediate diverse biological responses by binding growth factors and hormones termed ligands. In healthy tissue, a ligand dependent change in the oligomeric structure of the receptor turns these extracellular binding events into an intracellular signal, elevated kinase activity (reviewed in refs. (8-10)). Monomeric ligands, such as epidermal growth factor (EGF), cause dimerization by inducing a conformational change in the receptor that stabilizes its active dimeric form. Bivalent ligands mediate dimerization by crosslinking neighboring receptors. Accordingly, bivalent antibodies have been used to promote receptor dimerization by crosslinking neighboring receptors (11-13). Alternatively, overproduction of receptors promotes spontaneous dimerization (9, 14, 15) such that a common cellular lesion found in human cancers results from receptor overproduction and autocrine activation (15). Overexpression of growth factor receptors has been reported on breast, prostate, ovarian, bladder, pancreatic and lung cancers (4-7).

5.2. Role of ErbB family of receptors in malignant transformation

The epidermal growth factor (EGF) family consists of four (ErbB, ErbB2, ErbB3, and ErbB4) 185 kDa protein tyrosine receptors whose prototype is the EGF receptor (ErbB). These receptors consist of a 650 amino acid extracellular domain (ECD) with two characteristic cysteine-rich regions, a single transmembrane domain and an intracellular kinase domain. Members of this family have been implicated more than other growth factor receptors in the development of human adenocarcinomas. Increased expression of the EGF receptor (ErbB) and ErbB2 have been associated with aggressive tumors of the stomach, bladder, lung and breast. 20% of breast cancers are malignant by virtue of their abnormally high expression of EGFR and overexpression correlates with aggressive cancer and poor prognosis (6, 16, 17). Overexpression of either EGFR or ErbB2 correlates with poor prognosis (6, 14, 16-18). Moreover, data from numerous studies suggest that EGFR family members may participate in a complex yet flexible network of signal transduction by forming heterodimers between family members (19-21). These heterodimers may form even when only one receptor binds its ligand. Alternatively, cooperative signaling may explain why a specific ligand has not been identified which activates ErbB2. Cooperative signaling appears to play an important role in neoplastic transformation.

5.3. EGFR as a target for cancer therapy

Drugs which inhibit dimerization by members of the EGF family of receptors would have tremendous potential for arresting the growth of breast cancer. For example, Genentech Inc. has developed a humanized murine monoclonal antibody (Herceptin) which specifically inhibits proliferation of human tumor cells overexpressing ErbB2. This antibody is entering now an approved drug for breast cancer therapy (22). However many morphological and physiological features limit the accessibility of antibodies to tumors (23, 24) such that less than

0.01% of the injected antibody is localized in patients' tumors (25, 26). Thus development of small molecule inhibitors of EGFR extracellular domain (ECD) dimerization have greater long term promise for cancer therapy.

The traditional approach of screening chemical libraries for small molecule drug discovery has not yielded ErbB ECD dimerization antagonists. However, structure based drug design has led to promising new drugs (reviewed in refs. (27-31)). For example, knowledge of the three-dimensional structure of HIV protease has led to structure based design of protease inhibitors (32). Structure based receptor oligomerization inhibitors have also been identified (33-36). The feasibility of blocking ErbB ECD oligomerization is also evident from naturally occurring point mutations that abolish receptor dimerization and lead to a loss of function (37, 38). Taken together, these findings support the hypothesis that structure based drugs can block EGFR receptor dimerization to arrest growth of breast carcinomas.

5.4. The atomic structure of the EGFR extracellular domain (ECD)

Knowledge of the atomic structure of EGFR ECD would permit achievement of our goal of identifying small molecule EGFR dimerization antagonists capable of arresting cancer growth. For this work, we proposed to initiate work to determine the atomic structure of ErbB ECD by X-ray crystallography. Determination of the structural basis for ErbB dimerization is an essential step towards understanding the mechanism by which growth factor overexpression leads to unregulated growth. Insights gained into the mechanism of dimerization and signal transduction by analysis of the ErbB ECD structure may also be applicable to other members of the EGF family of receptors. As detailed above, solving the structure of EGFR ECD has tremendous translational potential for development of cancer therapy. Difficulties with conventional approaches include obtaining diffraction quality crystals and phase information. Obtaining crystals is frequently inhibited by heterogeneity and molecular flexibility which are likely to be hindrances with the large EGFR ECD glycoprotein. Another potential difficulty is finding suitable heavy atom derivatives which only occupy a limited number of sites and that do not cause major changes in the crystal unit cell dimensions. Our approach would use antibodies to obtain crystals by minimizing the molecular flexibility of EGFR ECD. Antibodies would also overcome the second difficulty with conventional approaches by allowing the introduction of a target for a limited number of heavy atom labels.

5.5. Purpose of the present work and methods of approach

Overproduction of EGFR on breast tissue leads to spontaneous EGFR ECD dimerization and rapid uncontrolled growth characteristic of tumor cells. Structure determination of EGFR ECD is the essential first step towards our goal to discover small molecules which inhibitor of EGFR ECD dimerization and tumor growth. We proposed to identify the EGFR ECD dimer interface by analyzing the atomic resolution structure of EGFR ECD. Our novel approach uses recombinant antibody fragments as tools to promote EGFR dimerization and to overcome the difficulties that have hindered structure determination of EGFR thus far. These obstacles include obtaining diffraction quality crystals and phase determination. To overcome these impediments, EGFR-antibody co-crystals will be grown. The antibodies will also be used as tools to promote receptor homo and heterodimerization to understand the complex role of receptor heterodimerization in signal transduction by the EGF family of receptors.

The proposed technical objectives in the statement of work were:

- Task 1: Overexpress and purify EGFR.
- Task 2: Isolate and identify recombinant antibodies which promote EGFR dimerization
- Task 3: Use recombinant antibody fragments to mediate EGFR crystallization
- Task 4: Obtain atomic resolution structural information on EGFR-diabody co-crystals
- Task 5: Construct heterodimeric bifunctional diabodies capable of binding EGFR and ErbB2 ECDs and evaluate the role of receptor heterodimerization in unregulated cell growth and signaling.

6. Body of report

6.1 Overexpress and purify EGFR

A stable CHO cell line expressing the EGFR extracellular domain (ECD) was obtained from Sugan. The cell line was grown in continuous culture in a CellMax in F12 media supplemented with 5% calf serum and supernatant containing EGFR ECD intermittently harvested from the CellMax. The EGFR ECD was purified on Concanavalin A with elution using 20 mM Hepes, pH 7.5, 2% glycerol and 0.5 M methyl- α -pyranoside. Eluate was concentrated using a Centricon and loaded onto a MonoQ column and eluted with a Hepes pH 7.5 gradient. After concentration, the EGFR ECD was > 95% pure as judged by SDS-PAGE.

6.2 Isolation and characterization of human anti-EGFR antibodies from phage display libraries

To generate a panel of EGFR antibodies, non-immune phagemid (32) and phage (33) antibody libraries were selected on recombinant purified EGFR ECD. After 2 to 3 rounds of selection, between 20 % and 90% of clones analyzed bound EGFR ECD by ELISA. The number of unique antibodies was determined by PCR fingerprinting followed by DNA sequencing, resulting in the identification of 62 unique antibodies. Each of these antibodies was expressed and purified and analyzed for binding to EGFR expressing cells and EGFR negative cells to identify those antibodies capable of binding native EGFR as expressed on the cell surface. This screening revealed that 30 of the 62 antibodies bound EGFR positive cells but not EGFR negative cells.

To generate additional EGFR antibodies capable of intracellular drug delivery, a non-immune phagemid library was directly selected on EGFR expressing cells. The approach used, in fact, selects for antibodies that not only bind to EGFR expressing cells but bind in a way that triggers receptor mediated endocytosis of the antibody. This selection is accomplished by first incubating phage antibodies with EGFR expressing cells at 37°C for 30 minutes and subsequently removing phage remaining on the cell surface by washing the cells extensively with low pH glycine buffer. Endocytosed phage antibodies are then recovered by lysis of the target cells. Phage antibodies are then amplified and the selection process is repeated. Using a model system employing an ErbB2 antibody, we previously demonstrated that phage antibodies can be endocytosed in a receptor dependent manner and that such endocytosed phage could be recovered from the cytosol and amplified (34). We also reported the successful application of this approach to generate internalizing antibodies to ErbB2 (35). One of these antibodies (F5) was efficiently endocytosed into ErbB2 expressing cells and was shown to be effective in delivering liposomes containing a fluorescent dye or the chemotherapeutic agent doxorubicin. Such antibodies are ideal for intracellular delivery of drugs to cancer cells for therapy. For example, we have shown in multiple mouse xenograft models that F5 ErbB2 targeted liposomal doxorubicin (F5 immunoliposomes, F5-ILs) has significant anti-tumor activity to an extent significantly greater than untargeted liposomal doxorubicin. Based on pre-clinical data, the National Cancer Institute (NCI) Decision Network elected to support clinical development of F5 ILs. To facilitate this, expression and purification of the F5 scFv antibody has been scaled to the 80L scale in a cGMP process at the NCI Monoclonal Antibody and Recombinant Protein (MARP) facility at Frederick MD. Toxicology studies and a phase 1 clinical trial are anticipated in early 2002.

To generate internalizing antibodies to EGFR capable of intracellular delivery of liposomes, a non-immune phage library was selected for endocytosis into either Chinese Hamster Ovary (CHO) cells transfected with the EGFR gene or into A431 tumor cells which overexpress EGFR (see Heitner et al, ref 36 and appendix 1). To remove antibodies binding common cell surface antigens, the library was incubated with CHO cells prior to selection. After 3 rounds of selection on A431 cells, 10/94 clones analyzed bound recombinant EGFR. After 3 rounds of selection on CHO cells transfected with the EGFR gene, 4/282 clones bound recombinant EGFR (Table 1, Heitner et al.). PCR fingerprinting and DNA sequencing revealed

2 unique antibodies from the A431 selections and 2 unique antibodies from the transfected CHO selections. One of these antibodies was common to both selections, yielding a total of 3 unique EGFR antibodies. To determine whether the monoclonal antibodies bound native EGFR as expressed on cells, phage and native scFv antibody were prepared from each of the three unique antibodies and used to stain cells which were analyzed by flow cytometry. Each of the antibodies stained EGFR expressing cells (A431, MDA-MB-468 and CHO cells transfected with the EGFR gene) but not EGFR negative cells (CHO cells and MDA-MB-453 cells) both as phage and as native scFv (Figure 3 and 4, Heitner et al.). The binding constants of the E12 antibody for EGFR was determined on A431 and MDA-MB-468 cells and determined to be 217 nM and 300 nM respectively. The ability of the antibodies to trigger EGFR mediated endocytosis was determined both for the phage antibodies as well as for native scFv. After incubation of EGFR expressing and EGFR negative cells with phage antibodies or native scFv, cells were fixed, surface phage or scFv removed with low pH glycine and intracellular phage detected with anti-M13 antibodies and intracellular scFv detected with an antibody to a C-terminal epitope tag. Both phage antibodies and native scFv were efficiently endocytosed into EGFR expressing cells but not into EGFR negative cells (figure 5 and 6, Heitner et al.). To determine the ability of the scFv to deliver a drug into EGFR expressing cells, immunoliposomes were constructed by inserting the E12 scFv into the surface of liposomes containing the fluorescent dye HPTS. Strong intracellular fluorescence was observed for EGFR expressing cells, but no fluorescence was observed for EGFR negative cells (figure 5 and 6, Heitner et al.). The results expanded the utility of this antibody selection methodology to transfected cell lines. Thus one route to generating internalizing antibodies to a known receptor would be to transfect the gene into a host cell and use the transfected cell for selection.

We are in the process of constructing doxorubicin containing ILs using the E12 scFv and will evaluate these in a xenograft model for anti-tumor activity. Similarly, we are screening scFv described above selected on recombinant EGFR for ability to trigger receptor mediated endocytosis. Antibodies which are efficiently endocytosed will be screened to identify the highest expressors and these will also be evaluated for anti-tumor activity in vivo. It is our intent to generate an EGFR immunoliposome from one of these scFv and develop it for a phase 1 clinical trials in EGFR expressing cancers as we have done for ErbB2 expressing cancers (see above).

6.3 Crystallize recombinant EGFR

Conditions were extensively screened to identify those conditions capable of mediating EGFR crystallization and then refined using additives. Conditions were identified which gave small crystals of several different morphologies (both in the presence and absence of EGF). These crystals were evaluated with respect to diffraction (6.4 below).

6.4 Obtain atomic resolution structural information on EGFR

Multiple crystals morphologies were investigated for their ability to diffract in the X-ray beam. Unfortunately, none of the crystal morphologies diffracted to high resolution. This included those crystals generated by co-crystallization with EGF or with the EGFR binding scFv E12. Pursuit of the crystallographic aspect of the project was hindered by the illness of the original Principal Investigator (Dr. Cara Marks) during the last 1.5 years of the grant, necessitating increased involvement of Dr. James Marks in the project. The original P.I. (Dr. Cara Marks) subsequently passed away and Dr. James Marks became the P.I. of the grant. As a result, emphasis was put on the generation and characterization of EGFR antibodies with therapeutic potential (section 6.2 above) consistent with the expertise of the new P.I.

6.5 Construct heterodimeric bifunctional diabodies capable of binding EGFR and ErbB2 ECDs

A heterodimeric diabody was constructed from the EGFR scFv E12 V_H and V_L genes and from the ErbB2 scFv C6.5. Expression levels of the diabody from *E. coli* were too low to generate adequate diabody for in vitro evaluation. As a result, a fermentation expression

system was developed which fuses a C-terminal cysteine to the scFv. This allows fermentation of individual scFv with a C-terminal cysteine. The scFv can then be conjugated to each other using a heterobifunctional agent. The ErbB2 scFv F5 was cloned into this expression system and expressed from *E. coli* RV308 with yields of 20 mg/L. The EGFR scFv E12 has been cloned into the same expression vector and will be fermented from *E. coli* RV308. These 2 scFv will then be linked to each other to create a bispecific scFv based antibody capable of binding EGFR and ErbB2 ECDs. The bispecific antibody will then be evaluated for in vitro activity. This approach will be used to generate additional bispecific scFv for in vitro evaluation.

7. Key research accomplishments

- Successful overexpression and purification of recombinant EGFR extracellular domain.
- Successful crystallization of recombinant EGFR extracellular domain.
- Generation of a panel of 33 single chain Fv antibody fragments binding EGFR extracellular domain.
- Development of a method for selecting internalizing antibodies to EGFR by selection of phage libraries on CHO cells transfected with the EGFR gene.
- Generation of 3 internalizing single chain Fv binding the EGFR which can be used for drug delivery to EGFR overexpressing tumors.

8. Reportable outcomes

8.1 Heitner T, Moor A, Garrison JL, Hasan T, and Marks JD. Selection of cell binding and internalizing epidermal growth factor receptor antibodies from a phage display library. *J. Immunol. Meth.* 248: 17-30, 2001.

8.2 Generation of a panel of human EGFR antibodies.

8.3 Funding successfully applied for based partially on work supported by this award: UCSF Prostate Cancer SPORE: NCI/NIH 1 P50 CA89520.

9. Conclusions

- 9.1. A high level expression system was developed from which the EGFR ECD could be expressed.
- 9.2. Despite adequate quantities of EGFR ECD, we were unable to obtain diffraction quality crystals, despite co-crystallization with EGF or an anti-EGFR scFv.
- 9.3. A large panel of human EGFR antibodies was generated by selection of phagemid and phage antibody libraries on recombinant EGFR ECD.
- 9.4. It is possible to directly select internalizing EGFR scFv antibodies directly on EGFR cell lines or CHO cells transfected with the EGFR gene.
- 9.5. This provides a route to generating internalizing antibodies to known cell surface receptors by transfecting the gene into an appropriate mammalian expression host. This provides a means of generating antibodies without the need for expressing and purifying protein.
- 9.6. Such antibodies are efficiently endocytosed as phage antibodies or as native scFv and can be used to deliver cytotoxic agents to the cytosol of tumor cells.
- 9.7. We are currently evaluating the anti-tumor potential of such antibodies by generating ILs from the E12 EGFR scFv

10. List of personnel receiving support:

Ruby Casareno Ph.D., Tara Heitner, Ph.D., Cara Marks Ph.D, Jennifer Pickering, Kaichi Sung, Puresh Maharaj, Ed Pickering, Kitty Sum

11. References

1. Brown, L., Miller, R.A., Horning, S.J., Czerwinski, D., Hart, S.M., Mcelderry, R., Basham, T., Warnke, R.A., Merigan, T.C., and Levy, R. (1989) Treatment of B-cell lymphomas with monoclonal anti-idiotypic antibodies alone and in combination with alpha-interferon. *Blood*. 73: 651-661.
2. Miller, R.A., Maloney, D.G., Warnke, R., and Levy, R. (1982) Treatment of B-cell lymphoma with monoclonal anti-idiotypic antibody. *Medical Intelligence*. 306: 517-522.
3. Barnd, D.L., Lan, M.S., Metzgar, R.S., and Finn, O.J. (1989) Specific, major histocompatibility complex-unrestricted recognition of tumor-associated mucins by human cytotoxic T cells. *Proc. Natl. Acad. Sci. USA*. 86: 7159-7163.
4. Ebert, M., Yokoyama, M., Friess, H., Korbin, M., Buchler, M., and Korc, M. (1995) Induction of platelet-derived growth factor A and B chains and over-expression of their receptors in human pancreatic cancer. *Int. J. Cancer*. 62: 529-535.
5. Di Blasio, A., Carniti, C., Viagano, P., and Vignali, M. (1995) Basic fibroblast growth factor and ovarian cancer. *J. Steroids Biochem. and Molec. Biol.* 53: 375-379.
6. Rachwal, W., Bongiorno, P., Orringer, M., Whyte, R., Ethier, S., and Beer, D. (1995) Expression and activation of erbB-2 and epidermal growth factor receptor in lung adenocarcinomas. *Brit. J. Cancer*. 72: 56-64.
7. Takahashi, Y., Kitadai, Y., Cleary, K., and Ellis, L. (1995) Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res*. 55: 3964-3968.
8. Ullrich, A. and Schlessinger, J. (1990) Signal Transduction by Receptors with Tyrosine Kinase Activity. *Cell*. 61: 203-212.
9. Wells, J.A. (1994) Structural and functional basis for hormone binding and receptor oligomerization. *Curr. Opin Cell Biol.* 6: 163-173.
10. Heldin, C. (1995) Dimerization of Cell Surface Receptors in Signal Transduction. *Cell*. 80: 213-223.
11. Davis, S., Gale, N.W., Aldrich, T.H., Maisonpierre, P.C., Lhotak, V., Pawson, T., Goldfarb, M., and Yancopoulos, G.D. (1994) Ligands for EPH-related receptor kinases that require membrane attachment or clustering for activity. *Science*. 266: 816-819.
12. Stancovski, I., Hurwitz, E., Ullrich, A., Yarden, Y., and Sela, M. (1991) Mechanistic aspects of the opposing effects of monoclonal antibodies to the ErbB2 receptor on tumor growth. *Proc. Natl. Acad. Sci. USA*. 88: 8691-8695.
13. Trauth, B.C., Klas, C., Peters, A.M., Matzku, S., Moller, P., Falk, W., Debatin, K.M., and Krammer, P.H. (1989) *Science*. 245: 301-305.
14. Slamon, D., Godolphin, W., and Jones, L. (1989) Studies of the HER2/neu proto-oncogene in human breast and ovarian cancer. *Science*. 244: 707-712.
15. Lemmon, M. and Schlessinger, J. (1994) Regulation of signal transduction and signal diversity by receptor oligomerization. *TIBS*. 19: 459-463.
16. Marcias, A., Azavedo, E., Hagerstorm, T., Perez, R., and Skoog, L. (1987) Prognostic significance of the receptor for epidermal growth factor receptor in human mammary carcinomas. *Anticancer Res*. 7: 459-464.
17. Seshadri, R., McLeay, W., Horsfall, D., and McCaul, K. (1996) Prospective study of the prognostic significance of EGFR in primary breast cancer. *Int. J. of Cancer*. 69: 23-7.

18. Slamon, D.J., Clark, G.M., Wong, S.G., Levin, W.J., Ullrich, A., and W.L., M. (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*. 235: 177-182.
19. Earp, H., Dawson, T., Li, X., and Yu, H. (1995) Heterodimerization and functional interaction between EGF receptor family members: a new signaling paradigm with implications for breast cancer research. *Breast Cancer Research and Treatment*. 35: 115-132.
20. Spivak-Kroizman, T., rotin, D., Pinchasi, D., Ullrich, A., Schlessinger, J., and Lax, I. (1992) Heterodimerization of c-erbB2 with different epidermal growth factor receptor mutants elicits stimulatory or inhibitory responses. *J. of Biol. Chem.* 267: 8056-8063.
21. Alimandi, M., Romano, A., Curia, M., Fedi, P., and Kraus, M. (1995) Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. *Oncogene*. 10: 1813-21.
22. Shepard, H., Lewis, G., and Sarup, J. (1991) Monoclonal antibody therapy of human cancer: taking the HER2 protooncogene to the clinic. *J. Clin. Immunol.* 11: 117-127.
23. Jain, R.K. (1987) Transport of molecules across tumor vasculature. *Cancer Met. Rev.* 6: 559-594.
24. Clauss, M.A. and Jain, R.K. (1990) Interstitial transport of rabbit and sheep antibodies in normal and neoplastic tissues. *Cancer Res.* 50: 3487-3492.
25. Colcher, D., Minelli, F.M., Roselli, M., Muraro, R., Simpson-Milenic, D., and Schlom, J. (1988) Radioimmunolocalization of human carcinoma xenografts with B72.3 second generation monoclonal antibodies. *Cancer Res.* 48: 4597-4603.
26. Gallinger, S., Reilly, R.M., Kirsh, J.C., Odze, R.D., Hay, K., Polihronis, J., Damani, M.T., Shpitz, B., and Stern, H.S. (1993) Comparative dual label study of first and second generation antitumor-associated glycoprotein-72 monoclonal antibodies in colorectal cancer patients. *Cancer Res.* 53: 271-278.
27. Kuntz, I. (1992) Structure-based Strategies for Drug Design and Discovery. *Science*. 257: 1078-1082.
28. Verlinde, C. and Hol, W. (1994) Structure-based drug design: progress, results and challenges. *Curr. Opin. in Structure Biol.* 2: 576-587.
29. Bugg, C., Carson, W., and Montgomery, J. (1993) Drugs by Design. *Scientific American*. 94-98.
30. Colman, P. (1994) Structure-based drug design. *Current Opinion in Structural Biology*. 4: 868-874.
31. Martin, Y. (1992) Database Searching in Drug Design. *J. of Med. Chem.* 35: 2147-2150.
32. Sheets MD, Amersdorfer P, Finnern R, Sargent P, Lindqvist E, Schier R, Hemingsen G, Wong C, Gerhart JC, Marks JD Panels of human antibodies without immunization from very large libraries of single chain Fv on phage. *Proc. Natl. Acad. Sci. USA*. 95: 6157-6162, 1998.
33. Huie MA, Cheung M-C, Muench, MO, Becerril B, Kan YW, Marks JD. Antibodies to human fetal erythroid cells from a non-immune phage antibody library. *Proc. Natl, Acad. Sci USA*. 98:2682-2687, 2001.
34. Becerril B, Poul M-A and Marks JD. Towards selection of internalizing antibodies from phage libraries. *Biochem. Biophys. Res. Comm.* 255: 386-393, 1999.
35. Poul, M-A, Becerril B, Nielsen UB, Morisson P, and Marks JD. Selection of tumor specific internalizing human antibodies from phage libraries. *J. Mol. Biol.* 301: 1149-1161, 2000.

36. Heitner T, Moor A, Hasan T, Garrison J, Marks C, and Marks JD. Selection of cell binding and internalizing epidermal growth factor receptor antibodies from a phage display library. J. Immunol. Meth. 248: 17-30, 2001.

12. Appendix One:

Heitner T, Moor A, Hasan T, Garrison J, Marks C, and Marks JD. Selection of cell binding and internalizing epidermal growth factor receptor antibodies from a phage display library. J. Immunol. Meth. 248: 17-30, 2001.



ELSEVIER

Journal of Immunological Methods 248 (2001) 17–30

JIM
Journal of
Immunological Methods

www.elsevier.nl/locate/jim

Selection of cell binding and internalizing epidermal growth factor receptor antibodies from a phage display library

Tara Heitner^a, Anne Moor^b, Jennifer L. Garrison^a, Cara Marks^a, Tayyaba Hasan^b,
James D. Marks^{a,*}

^aDepartments of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco, Room 3C-38,

San Francisco General Hospital, 1001 Potrero Avenue, San Francisco, CA 94110, USA

^bWellman Laboratories of Photomedicine, Harvard Medical School, Massachusetts General Hospital, 50 Fruit Street, Boston, MA 02114, USA

Abstract

The first step in developing a targeted cancer therapeutic is generating a ligand that binds to a receptor which is either tumor specific or sufficiently overexpressed in tumors to provide targeting specificity. For this work, we generated human monoclonal antibodies to the EGF receptor (EGFR), an antigen overexpressed on many solid tumors. Single chain Fv (scFv) antibody fragments were directly selected by panning a phage display library on tumor cells (A431) overexpressing EGFR or Chinese hamster ovary cells (CHO/EGFR cells) transfected with the EGFR gene and recovering endocytosed phage from within the cell. Three unique scFvs were isolated, two from selections on A431 cells and two from selections on CHO/EGFR cells. All three scFv bound native receptor as expressed on a panel of tumor cells and did not bind EGFR negative cells. Phage antibodies and multivalent immunoliposomes constructed from scFv were endocytosed by EGFR expressing cells as shown by confocal microscopy. Native scFv primarily stained the cell surface, with less staining intracellularly. The results demonstrate how phage antibodies binding native cell surface receptors can be directly selected on overexpressing cell lines or transfected cells. Use of a transfected cell line allows selection of antibodies to native receptors without the need for protein expression and purification, significantly speeding the generation of targeting antibodies to genomic sequences. Depending upon the format used, the antibodies can be used to deliver molecules to the cell surface or intracellularly. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Receptor mediated endocytosis; Epidermal growth factor receptor; Phage antibody library; Single chain Fv; scFv; Tumor targeting

1. Introduction

Traditional cancer therapies have relied on the differential toxicity of chemotherapeutic agents on tumor cells compared to normal cells. Recently,

improved understanding of the molecular basis of cancer makes possible the development of therapies with increased efficacy and reduced toxicity. Studies of tumorigenesis have identified cell surface receptors which are either tumor or lineage specific, such as CD20 (Einfeld et al., 1988) and mutant forms of epidermal growth factor receptor (EGFR) (Garcia de Palazzo et al., 1993) or receptors which are over-

*Corresponding author.

E-mail address: marksj@anesthesia.ucsf.edu (J.D. Marks).

expressed in tumors, such as ErbB2 (Slamon et al., 1989). Such receptors can be targeted with antibodies to allow specific drug interaction with only the tumor cell. In some instances, binding of “naked” antibody to the tumor cell can cause growth inhibition (Carter et al., 1992) or apoptosis (Ghetie et al., 1997; Taji et al., 1998). Alternatively, the antibody can be used to deliver a “toxic payload” to the cell. Toxic mechanisms include activation of the immune system, e.g., with bispecific antibodies, fusions to co-stimulatory molecules, or fusion with a toxic payload including radioisotopes, chemotherapeutics, toxins, or genes. For some strategies, it is necessary for the antibody to remain on the cell surface (e.g., bispecific therapies). For other approaches, it is necessary that the antibody deliver its payload into the cytosol (e.g., immunotoxins and gene therapy). In both cases, antibody recognition of the native receptor as expressed on the cell surface is required.

Phage antibody libraries have become an important source for the development of completely human therapeutic antibodies (Marks and Marks, 1996; Marks et al., 1991) to a wide range of antigens including tumor growth factor receptors (Schier et al., 1995). Antibodies generated from phage libraries have typically been selected using purified antigens or peptides immobilized on artificial surfaces. This approach may select antibodies that do not recognize the native protein in a physiologic context, as on the surface of cells. Attempts have been made to select on antigen in native conformation using cell lysates (Parren et al., 1996; Sanna et al., 1995; Sawyer et al., 1997) fixed cells (Van Ewijk et al., 1997) or living cells (Andersen et al., 1996; Cai and Garen, 1995; de Kruif et al., 1995; Marks et al., 1993; Siegel et al., 1997). The few successful selections performed on such heterogeneous material were generally done using small libraries from immunized sources. The use of immunized libraries limits the spectrum of antigen specificities that can be potentially obtained from the same library and typically yields murine antibodies. Selection of binders from large naïve libraries by cell panning is greatly limited by high background binding of non-specific phage and relatively low binding of specific phage (Pereira et al., 1997; Watters et al., 1997; Becerril et al., 1999).

Using a model system and an ErbB2 phage

antibody we recently demonstrated that phage antibodies binding internalizing surface receptors can be endocytosed by mammalian cells and recovered in infectious form from within the cell (Becerril et al., 1999). Enrichment of ErbB2 phage over non-specific phage was 10–30 times higher when phage were recovered from within the cell compared to recovery from the cell surface, suggesting that cell selection specificity could be increased by recovering internalized phage. We confirmed this by applying this methodology to generate a panel of anti-tumor antibodies which were endocytosed into the breast tumor cell line SKBR3 as well as other tumor cells (Poul et al., 2000). Two of the specificities isolated included ErbB2 and transferrin receptor antibodies.

For this work, we applied the methodology to generate EGFR antibodies which recognized the native receptor on cells and could be used for tumor targeting. EGFR is overexpressed in many carcinomas (Baselga and Mendelsohn, 1994; Chrysogelos and Dickson, 1994; De Jong et al., 1998; Harris, 1994; LeMaistre et al., 1994) and can be exploited to differentiate and target cancer cells from normal cells. For selections, two cell lines were used as the source of antigen: a transfected Chinese hamster ovary cell (CHO/EGFR) and EGFR-over-expressing cancer cell line A431. The results indicate the generality of the approach and its usefulness in generating antibodies to known receptors in the absence of purified recombinant protein.

2. Materials and methods

2.1. Cell culture

CHO cells stably transfected with EGFR full length receptor (Morrison et al., 1993) (CHO/EGFR) were grown in F12 selective media (G418, Mediatech, 0.8 g/l) supplemented with 10% fetal calf serum (FCS). The parent cell line (CHO) was grown in non-selective F12 complete media supplemented with 10% FCS. A431 cells were grown in DMEM supplemented with 10% FCS. MDA-MB-453 and MDA-MB-468 cells were grown in Leibovitz media supplemented with 10% FCS in the absence of CO₂. All other cell lines were grown at 37°C in the presence of 5% CO₂.

2.2. Phage antibody selections

2.2.1. Selections on CHO/EGFR cell monolayer

CHO/EGFR cells grown on a 10-cm plate at 80–90% confluence were incubated with 1 ml of phage antibody library (5×10^{12} cfu/ml) (Sheets et al., 1998) in the presence of 2×10^6 CHO cells in complete media (3 ml) for 1.5 h at 4°C. CHO cells were used to deplete the library of non-specific clones. The supernatant was aspirated and cells were washed six times in cold complete media for 10 min per wash. Receptor internalization was induced by addition of pre-warmed (37°C) complete media and incubation at 37°C, 5% CO₂ for 15 min. This time period has been shown to be appropriate for the observation of EGFR internalization (Vieira et al., 1996). After internalization, non-internalized cell-membrane bound phage were eluted by washing cells on the plate with cold glycine buffer (50 mM glycine, 150 mM NaCl, 200 mM urea, 2 mg/ml polyvinylpyrrolidone, pH 2.8) three times for 10 min per wash at 4°C. Immobilized cells were washed 1× in complete media. The internalized phage were recovered by removing cells in trypsin and washing in complete media. Cells were pelleted by centrifugation at 1000 rpm, lysed in 0.5 ml 100 mM triethylamine (TEA) for 10 min and neutralized in 1 ml 1 M Tris, pH 7.

2.2.2. Selections on A431 cells in suspension

A431 cells growing on a 15-cm culture dish (90% confluence) were removed in 2 mM EDTA–phosphate-buffered saline (PBS) and washed twice in cold PBS (25 ml). To deplete the library of non-specific phage, 5×10^6 fibroblast cells (ATCC, CRL1634) were incubated with 1 ml of phage antibody library in 3 ml complete media (DMEM–10% FCS) for 1 h rocking at 4°C. Fibroblast cells were pelleted by centrifugation at 1000 rpm and the supernatant was recovered. A431 cells were incubated in a 15-ml culture tube with the depleted phage antibody library (supernatant from the previous step) for 1.5 h rocking at 4°C. Cells were subsequently washed 10 times in cold complete media. Cells were incubated for 30 min at 37°C in pre-warmed complete media to allow receptor internalization. Non-internalized phage were removed from the cell surface by 10 washes in cold PBS and a final wash in

glycine buffer. Cells were lysed immediately, following a single glycine wash, in 0.5 ml 100 mM TEA and neutralized in 1 ml 1 M Tris, pH 7.

2.3. Phage rescue, preparation and titration

Phage were titered by infection of eluted phage into *Escherichia coli* TG1 (Marks et al., 1991). Phage were prepared for the next round of selection by infection of *E. coli* TG1 with eluted phage and rescue with VCS-M13 (Stratagene) helper phage as previously described (Marks et al., 1991). After overnight growth at 30°C, phage were purified and concentrated from bacterial supernatant with polyethylene glycol 8000 (PEG8000) (Marks et al., 1991) and resuspended in 1.5 ml PBS for use in the next round of selection or for use in flow cytometry. For each cell type, a total of three rounds of selection were performed.

2.4. Polyclonal phage enzyme-linked immunosorbent assay (ELISA)

EGFR-ECD was expressed in CHO cells and purified by concavalin A agarose (Vector Laboratories) affinity chromatography. Ninety-six-well microtiter plates (Falcon, 353912) were coated overnight at 4°C with 10 µg/ml of EGFR-ECD in PBS. Plates were washed three times with PBS and 50 µl of 1.0×10^{11} cfu/ml of polyclonal phage in PBS buffer (prepared as described in Section 2.3) was added to each well and incubated for 1 h. Wells were washed three times with PBS containing 0.1% Tween 20 (TPBS) and three times with PBS. Binding of phage antibodies was detected with peroxidase-conjugated anti-M13 antibody (Amersham-Pharmacia) diluted 1:1000 in PBS and ABTS (Sigma) as substrate.

2.5. Evaluation of polyclonal phage mixtures by flow cytometry

Polyclonal phage were screened for binding to whole cells by fluorescence activated cell sorting (FACS) analysis. EGFR expressing cell lines MDA-MB-468 and CHO/EGFR were used to identify EGFR binding antibodies and EGFR negative cell lines MDA-MB-453 and CHO were used to de-

termine specificity. Cells were grown to 80–90% confluence and removed in 2 mM EDTA–PBS. Cells were counted and washed once in cold PBS and twice in FACS buffer [cold 0.5% bovine serum albumin (BSA) (fraction V, Sigma)–PBS]. Cells were placed in FACS tubes (100 000 cells/well) and incubated with polyclonal phage ($50 \mu\text{l}/10^{12}$ cfu/ml) for an hour on ice. Cells were washed twice in FACS buffer and incubated with α -M13-biotin (Amersham-Pharmacia, 1:5000 dilution) for 30 min on ice. Cells were washed twice in FACS buffer and incubated with streptavidin-PE (Biosource International, 1:1000 dilution) for 30 min on ice. Cells were washed twice in FACS buffer and then analyzed by FACS on the PE channel. Fluorescence was measured in a FACSsort™ (Beckton Dickinson) and mean fluorescence was calculated using the Cellquest™ software.

2.6. Isolation and characterization of monoclonal EGFR antibodies

To facilitate subsequent purification of soluble single chain Fv (scFv), the polyclonal scFv gene population from the third round of selection was subcloned in batch into the expression vector pUC119mycHis (Schier et al., 1995) resulting in the addition of a c-myc epitope tag and hexahistidine tag at the C-terminus of the scFv. Briefly, phagemid DNA was prepared from the third round of selection, the scFv genes excised using the restriction enzymes *Sfi*I and *Not*I, and the gene repertoire gel purified and ligated into pUC119mycHis digested with *Sfi*I and *Not*I. After transformation of *E. coli* TG1, single ampicillin resistant colonies were packed into 96-well microtiter plates and scFv expression induced by the addition of IPTG as previously described (Schier et al., 1996). Bacterial supernatant containing scFv was used directly for ELISA. For EGFR-ECD ELISA using unpurified scFv, microtiter plates (Falcon) were coated with $10 \mu\text{g}/\text{ml}$ EGFR-ECD in PBS overnight at 4°C . Plates were incubated with bacterial supernatants at room temperature for 1 h and then washed once in TPBS and twice in PBS. Protein binding was detected with anti-myc tag antibody 9E10 followed by incubation in secondary antibody anti-mouse-horseradish peroxidase (HRP) (Sigma)

for 30 min as previously described (Schier et al., 1996). Following a final set of washes, binding was detected with ABTS substrate. For further studies of monoclonal scFv, expression from pUC119 mycHis was scaled up into 500-ml cultures in 2 L culture flasks. Cultures were grown and scFv expressed (De Bellis and Schwartz, 1990) as previously described (Schier et al., 1996). scFv was harvested from the bacterial periplasm by osmotic shock (Breitling et al., 1991) and purified by immobilized metal affinity chromatography (Hochuli, 1988) using a Ni-NTA column (Qiagen) and gel filtration, as previously described (Schier et al., 1996).

2.7. Covalent labeling of α -EGFR scFv with fluoroisothiocyanate (FITC)

A 1-ml (1 mg) volume of E12 scFv was dialyzed against 50 mM carbonate buffer, pH 8.5 overnight. Fluoroisothiocyanate (FITC) labeling reagent, 6-(fluorescein-5-[and-6]-carboxamido)hexanoic acid, succinimidyl ester [5(6)-SFX, Molecular Probes] was dissolved in dimethylsulfoxide (DMSO) or dimethylformide (DMF) (5–10 mg/ml) and added to the scFv at a volume:volume ratio of 1:20. The reaction was conducted for 1 h at room temperature. Free labeling reagent was separated from labeled antibody on a S-25 gel filtration column (Sephadex). E12 scFv was incubated with CHO cells and CHO/EGFR cells and no background binding due to free label could be detected. No fluorescence shift was detected for CHO cells stained with the labeled antibody. The fluorescence shift detected on CHO/EGFR cells was therefore wholly attributed to the antibody–receptor interaction.

2.8. Microscopy

2.8.1. Fixed cell microscopy

Confirmation of α -EGFR phage antibody internalization was obtained by confocal microscopy. Cells were grown on coverslips in 24-well plates and at 80% confluence were incubated with phage antibody (10^{10} cfu/ml, in fresh complete media) for 2 h at 37°C . Plates were placed on ice to halt receptor internalization and the coverslips washed 10 times in cold PBS (1 ml per wash). Cells were then washed three times for 10 min in cold glycine buffer, pH 2.8

(50 mM glycine, 150 mM NaCl) to remove surface bound antibody. Cells were washed twice in cold PBS, fixed in 4% paraformaldehyde for 10 min at room temperature and then permeabilized in cold methanol for 10 min at room temperature. Coverslips were incubated with α -M13-biotin (5'–3', 1:2000 dilution in 0.5% BSA–PBS) for 30 min followed by two washes in complete media. Phage were detected by incubation with streptavidin-PE (Biosource International, 1:1000 dilution) for 30 min while followed by two washes in complete media. Coverslips were mounted on microscope slides and 2–3 μ l Vectashield (Vector Laboratories) was applied to each coverslip to preserve fluorescence upon irradiation.

2.8.2. Live cells

scFv-mediated internalization of FITC-labeled soluble-native scFv (100 μ g/ml) or fluorescent immunoliposomes was measured on live cells grown on coverslips. E12 scFv immunoliposomes were constructed as previously described and contained on average 25 scFv/liposome (Park et al., 1998). Cells were plated and grown overnight on coverslips to 80% confluency. Cells were incubated with 100 μ g/ml FITC-labeled (0.5 ml volume) or unlabeled scFv or with 10 μ M immunoliposomes for 2 h. Cells were washed with PBS, coverslips removed and mounted onto microscope slides for imaging. Images were collected immediately using a Leica TCS NT confocal laser fluorescence microscope with digital camera (Leica, Deerfield, IL, USA).

2.9. Affinity measurement on whole cells by FACS

Cells (A431, MDA-MB-468, MDA-MB-453) were grown to 90% confluence in DMEM (A431) and Leibovitz (MDA-MB) media supplemented with 10% FCS. Cells were harvested in 2 mM EDTA–PBS. scFv was incubated with 2.5×10^5 cells for an hour at varying concentrations (50 nM–2 μ M). Cell binding was performed on ice in PBS containing 0.25% BSA in a total volume of 100 μ l. After two washes in PBS–BSA (250 μ l), cells were incubated with saturating amounts of anti-myc 9E10 for 30 min followed by two washes in PBS–BSA. Bound scFv was detected by staining with saturating amounts of anti-mouse FITC, Fc specific (1:200 dilution, Sigma). After a 30 min incubation, cells were

washed twice and resuspended in PBS containing 1% paraformaldehyde. Determination of the binding affinity was determined using a flow cytometry based assay as previously described (Benedict et al., 1997).

3. Results

3.1. Selection of EGFR antibodies

For selections, phage were prepared from a 7.0×10^9 member human scFv phage antibody library (Sheets et al., 1998). To generate antibodies binding EGFR, phage was selected on the A431 cell line which overexpresses EGFR and on CHO cells transfected with the EGFR gene (CHO/EGFR). For selections on CHO/EGFR cells, the library was depleted of antibodies binding common cell surface receptors by adding phage to CHO/EGFR cells grown adherent to subconfluency with untransfected CHO cells in suspension. After 1 h at 4°C, CHO cells were removed from the culture flask and warm media at 37°C added to allow internalization into the target CHO/EGFR cells. For selections on A431 cells, which grow in suspension, the phage library was pre-depleted of antibodies binding common cell surface receptors by incubation with fibroblast cells. After 1 h at 4°C, the fibroblast cells were removed by centrifugation and the phage added to A431 cells in suspension at 4°C to allow binding followed by incubation at 37°C to allow phage internalization. After phage endocytosis, cells were extensively washed and then lysed with TEA. The cell lysate containing the internalized phage was used to infect *E. coli* to prepare phage for the next round of selection. Three rounds of selection were performed with the efficiency of selection monitored by titering the number of phage recovered from the cell lysate. For selections on both A431 and CHO/EGFR cells, the titer of phage recovered increased with each round of selection, consistent with enrichment for cell binding antibodies (Table 1).

3.2. Analysis of polyclonal phage for EGFR binding by ELISA and FACS

To evaluate the success of selections, polyclonal phage was prepared after each round of selection and

Table 1
Results of selection of α -EGFR scFv on whole cells in three rounds

Round	A431		CHO/EGFR	
	Phage titer (cfu)	Frequency of positives	Phage titer (cfu)	Frequency of positives
R1	1×10^4	ND	2×10^3	ND
R2	8×10^5	ND	8×10^4	ND
R3	8×10^6	10/94	5×10^6	4/282

analyzed for binding to recombinant EGFR-ECD by ELISA (Fig. 1). A signal significantly greater than background binding was observed after three rounds of selection on both A431 and CHO/EGFR cells (Fig. 1). No significant binding above background was observed after one or two rounds of selection on either cell type. Binding of polyclonal phage from the third round of selection to cell lines expressing different quantities of EGFR was studied further by flow cytometry. Phage selected on A431 cells showed a significantly greater fluorescent shift on CHO/EGFR cells than on CHO cells (Fig. 2 left panels) and on the high EGFR expressing tumor cell line MDA-MB-468 vs. the EGFR negative tumor cell line MDA-MB-453 (Fig. 2 left panels). For phage selected on CHO/EGFR cells, no significant difference in fluorescent shift was observed for binding to

CHO/EGFR cells vs. CHO cells (Fig. 2 right panels). The strong shift on both cell lines indicates that the majority of the phage bind antigens common to CHO cells. Analysis of these phage for binding to high EGFR expressing MDA-MB-468 cells compared to EGFR negative MDA-MB-453 cells indicates, however, the presence of a relatively small number of phage binding EGFR (Fig. 2, right panels).

3.3. Isolation and characterization of monoclonal EGFR antibodies

Based on the ELISA and flow cytometry data indicating the presence of EGFR phage antibodies, individual clones were picked into 96-well microtiter plates and expression of native soluble scFv induced. Bacterial culture supernatants containing scFv were analyzed by ELISA for their ability to bind recombinant EGFR-ECD. For the third round of selection on A431 cells, 10/94 clones (11%) bound EGFR-ECD, while for the third round of selection on CHO/EGFR cells, 4/282 (1%) clones bound EGFR-ECD (Table 1). The relative proportions of binders is consistent with the ELISA and flow cytometry analysis of the polyclonal phage. To determine the number of unique antibodies, the scFv gene of all EGFR-ECD binding clones was analyzed by BstN1 fingerprinting followed by DNA sequencing. Two unique EGFR antibodies (E12 and B11) were isolated from selections on A431 cells. For selections on CHO/EGFR cells, the B11 scFv was re-isolated along with another unique scFv (C10).

To determine whether the monoclonal antibodies bound native EGFR as expressed on cells, phage and native scFv were prepared from each of the three

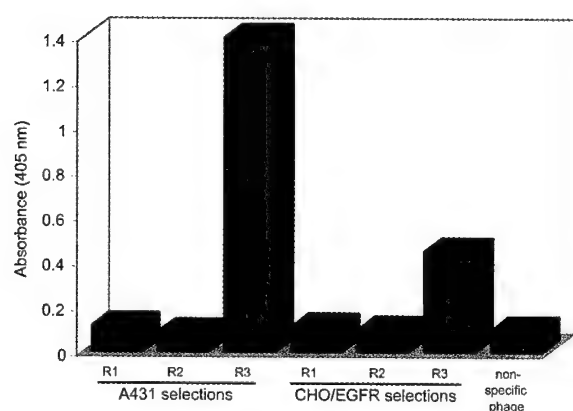


Fig. 1. Binding of polyclonal phage to recombinant EGFR as determined by ELISA. Phage was prepared from the first, second and third round of selections and analyzed for binding to recombinant EGFR by ELISA. After the third round of selection, binding was observed for selections performed on A431 cells and for selections performed on CHO/EGFR cells.

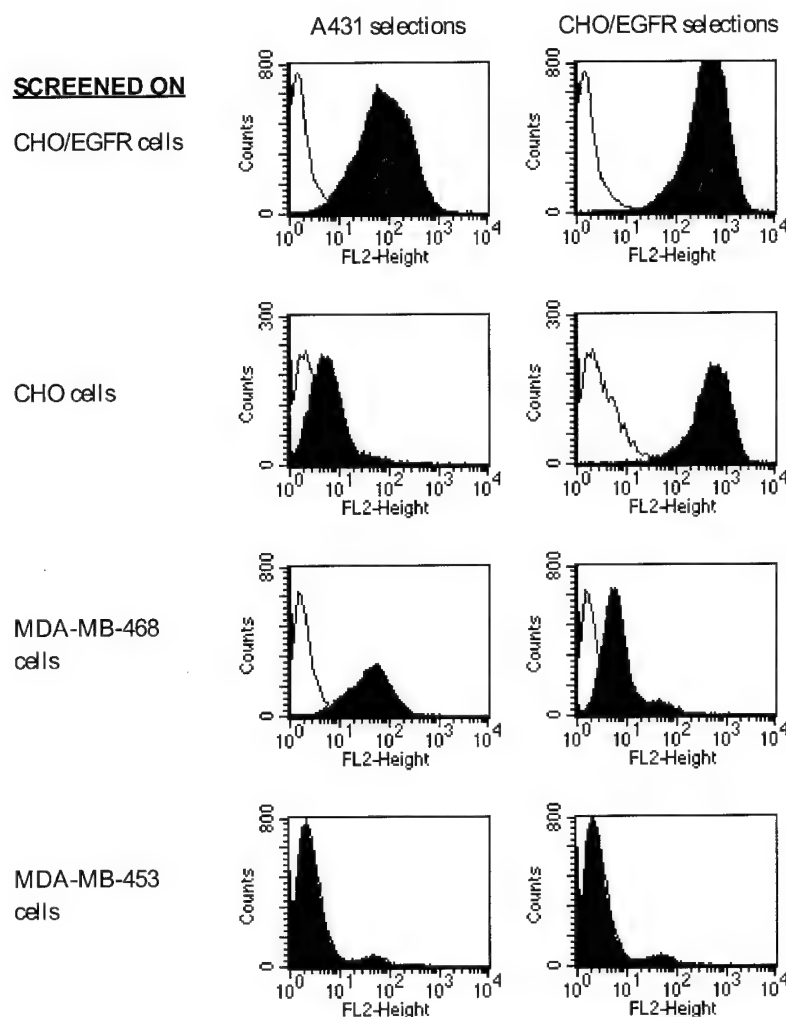


Fig. 2. Binding of polyclonal phage to EGFR expressing cells. Phage was prepared from the third round of selections performed on A431 cells and CHO/EGFR cells, and binding to a panel of cells was analyzed by flow cytometry. For selections performed on A431 cells (left panels), phage stained EGFR expressing cells (CHO/EGFR and MDA-MB-468 cells) more strongly than EGFR negative cells (CHO and MDA-MB-453 cells). For selections performed on CHO/EGFR cells (right panels), no difference in staining was observed between CHO/EGFR cells and CHO cells, however EGFR expressing cells stained more intensely than EGFR negative cells. This result suggests that many antibodies were selected that bind antigens common to CHO cells with a minority of antibodies binding EGFR.

unique scFvs and used to stain cells which were analyzed by flow cytometry. Each of the monoclonal antibodies stained EGFR expressing cells (A431, MDA-MB-468 and CHO/EGFR) but not EGFR negative cells (MDA-MB-453 and CHO) both as phage antibodies (Fig. 3) and as native scFv (Fig. 4). The binding constant for EGFR of each of the native scFvs was determined on A431 cells and on MDA-

MB-468 cells (for the E12 scFv). The K_D values ranged between 217 and 300 nM (Table 2).

3.4. Cell binding and internalization of phage antibodies and scFv

Since the phage antibodies were selected on the basis of internalization, we examined the ability of

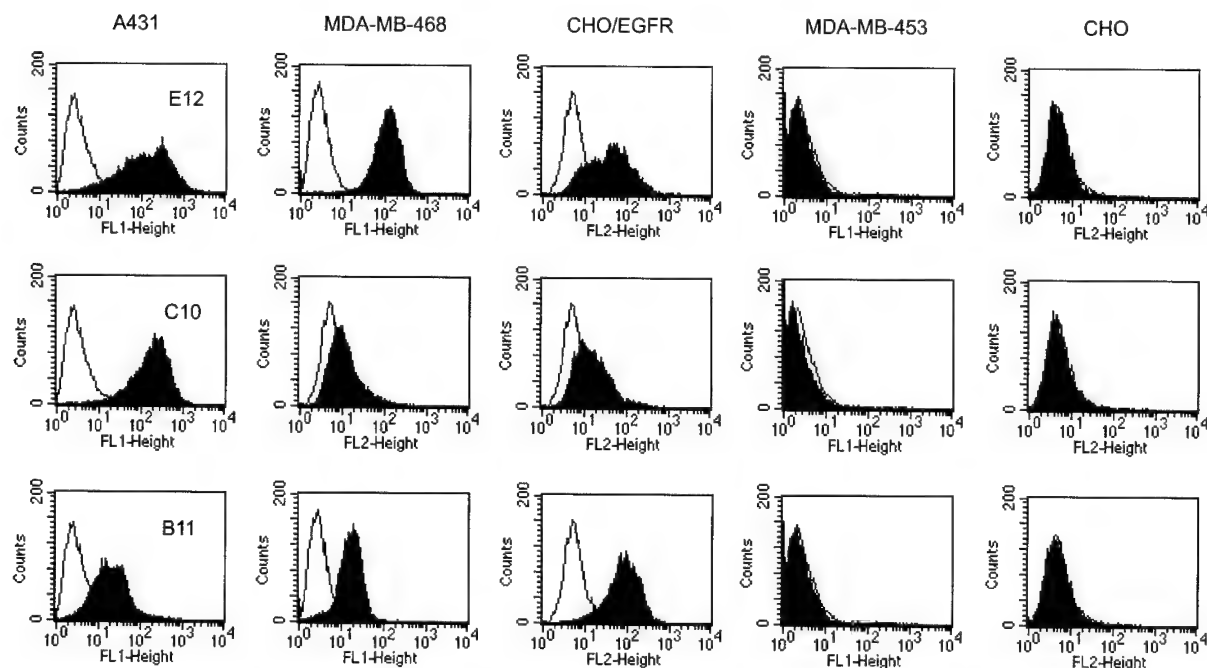


Fig. 3. Binding of monoclonal phage to EGFR positive and negative cell lines. Phage antibodies (E12, C10 and B11) were analyzed for the ability to bind EGFR positive (A431, MDA-MB-468 and CHO/EGFR) and EGFR negative (MDA-MB-453 and CHO) cell lines by flow cytometry. All three antibodies stained EGFR expressing cells and did not stain EGFR negative cells.

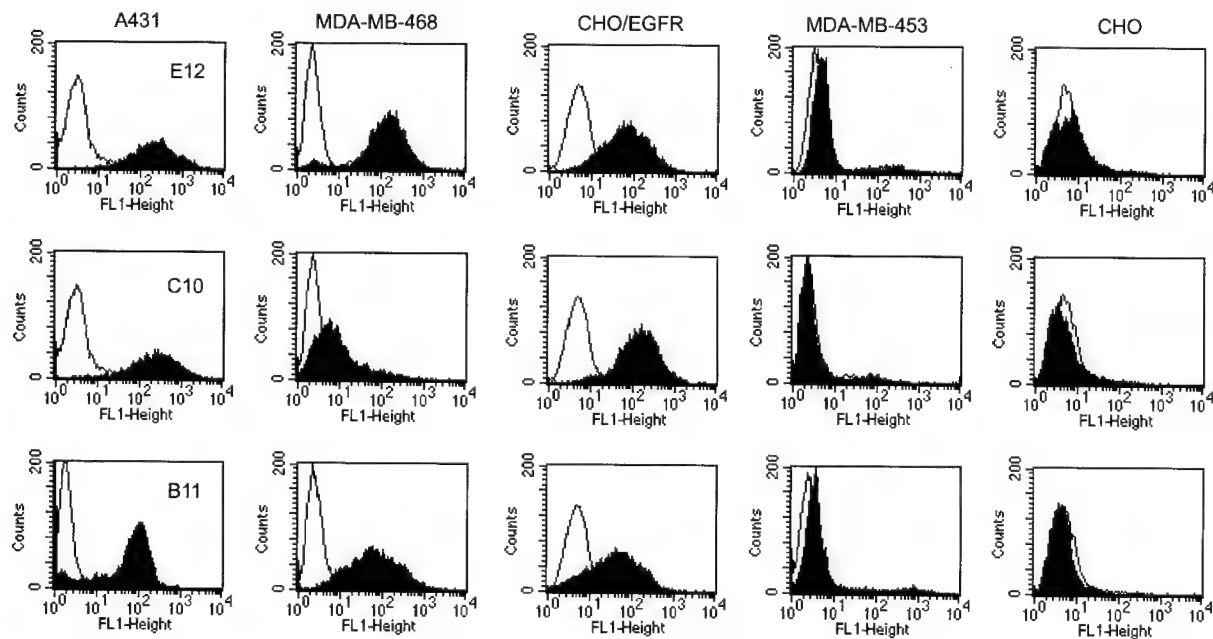


Fig. 4. Binding of monoclonal scFv to EGFR positive and negative cell lines. Purified scFvs (E12, C10, and B11) were analyzed for the ability to bind EGFR positive (A431, MDA-MB-468 and CHO/EGFR) and EGFR negative (MDA-MB-453 and CHO) cell lines by flow cytometry. All three antibodies stained EGFR expressing cells and did not stain EGFR negative cells.

Table 2
Binding affinity of α -EGFR scFv

scFv	K_D (nM)	
	A431	MDA-MB-468
E12	300	265
C10	217	–
B11	280	–

the phage antibodies to be endocytosed by EGFR expressing cells. After incubation of EGFR expressing and EGFR negative cells with phage antibodies, cells were fixed, surface phage removed with low-pH glycine and intracellular phage detected with anti-M13 antibody and confocal microscopy. Intracellular phage were detected in EGFR expressing cells (e.g., A431, MDA-MB-468 and CHO/EGFR) but not in EGFR negative cells (MDA-MB-453 and CHO). Representative results are shown for the E12 scFv on CHO/EGFR and CHO cells (Fig. 5) and on the tumor cell lines MDA-MB-468 and MDA-MB-453 (Fig. 6). To determine if native scFv were endocytosed by EGFR expressing cells, scFv were directly FITC labeled and incubated with live cells. After incubation, cells were analyzed directly by confocal microscopy allowing observation of surface bound and intracellular scFv. Staining of EGFR expressing cells was observed (e.g., A431, MDA-MB-468 and CHO/EGFR) but no staining was seen for EGFR negative cells (MDA-MB-453 and CHO). Much of the scFv remained surface bound, with some intracellular staining observed (Figs. 5 and 6 for representative results with E12 scFv). To determine the ability of the scFv to deliver a drug to EGFR expressing cells, immunoliposomes were constructed by fusing E12 scFv to the surface of HPTS containing liposomes. Strong intracellular fluorescence was observed for EGFR expressing cells, with no fluorescence observed for EGFR negative cells.

4. Discussion

The first step in developing a targeted cancer therapeutic is generating a ligand that specifically binds to a receptor which is either tumor specific or sufficiently overexpressed in tumors to provide targeting specificity. Antibodies have proved to be

important targeting ligands for cell surface receptors, especially with recent engineering techniques to generate antibodies which are entirely human in sequence. Libraries of antibodies displayed on phage can rapidly generate panels of human antibodies to a target antigen without the need for immunization (Marks et al., 1991; Sheets et al., 1998). To generate antibodies which bind native cell surface receptors, we recently demonstrated that phage could be directly selected on tumor cell lines by recovering endocytosed phage from within the target cell (Poul et al., 2000). Compared to simply recovering phage from the cell surface, intracellular phage recovery increases specific enrichment of antigen binding antibodies more than 10- to 30-fold (Becerril et al., 1999). High enrichment ratios are essential for successful selection of antibodies on heterogeneous antigens such as the surface of cells. In our previous publication, more than 10 unique tumor specific antibodies were generated, two of which were determined to bind ErbB2 and the transferrin receptor (Poul et al., 2000).

For this work, we demonstrate that this approach can be used to generate human scFv antibodies to a known tumor antigen (EGFR). EGFR is a 170-kDa transmembrane glycoprotein overexpressed in a number of human cancers. Ligand binding induces receptor dimerization which results in autophosphorylation of the kinase domain (Odaka et al., 1997; Tzahar et al., 1997). Receptor internalization occurs following dimerization and is believed to be a mechanism of receptor signal downregulation. EGFR antibodies were generated both by selecting on an overexpressing cell line or by using a cell line transfected with the target gene. The transfected human EGFR has been shown to function normally in its foreign environment: stimulation with EGF leads to receptor phosphorylation and receptor internalization follows activation. Selection on the EGFR transfected cell line permits use of the untransfected parental cell line to deplete the library of phage binding irrelevant receptors, enhancing enrichment ratios. The two cell lines should differ only in the presence of the target receptor. The availability of the untransfected cell line also provides an ideal reagent for the screening and characterization of antigen specific clones following selection. The ability to select on a transfected cell also eliminates

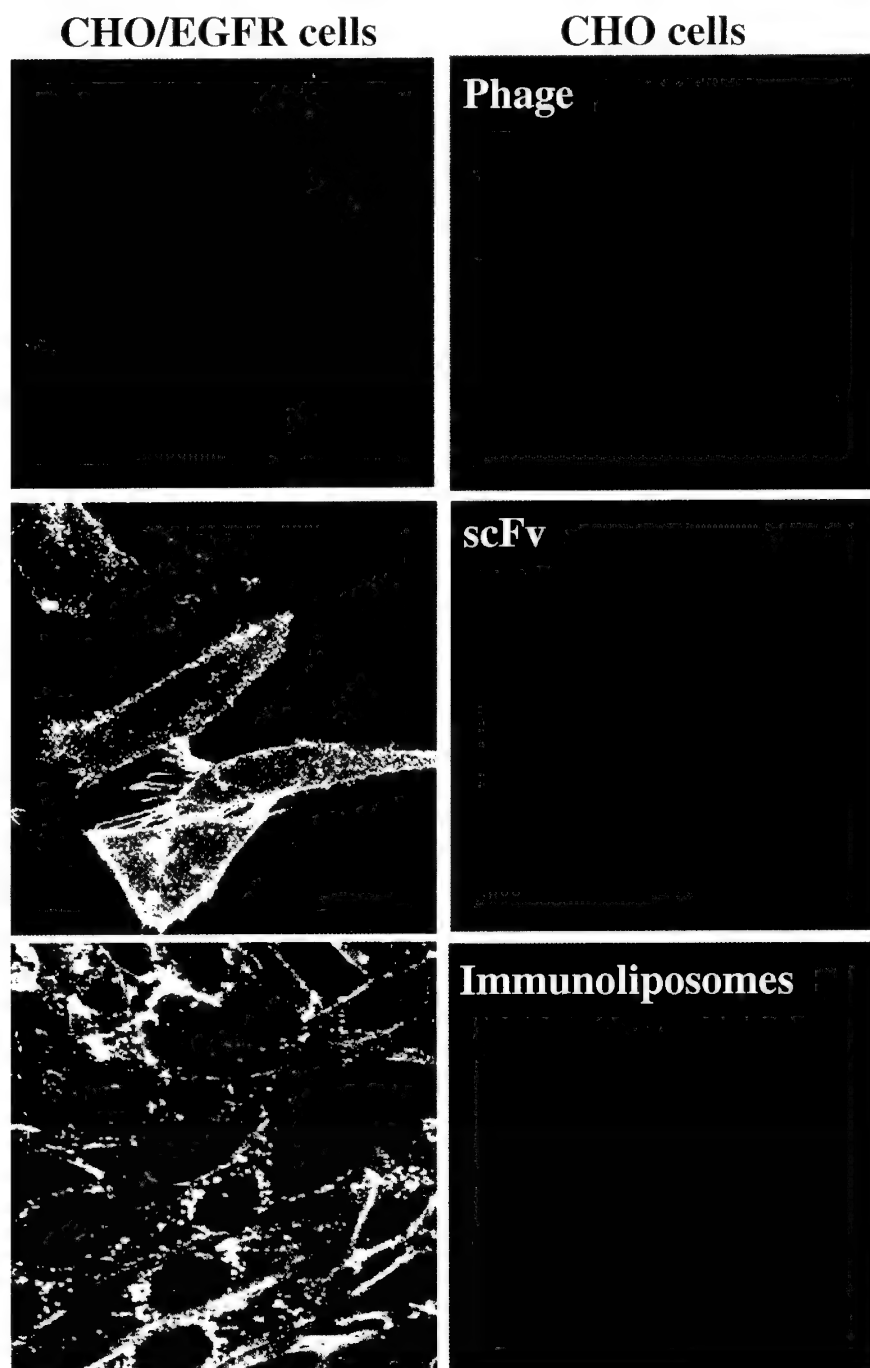


Fig. 5. Binding and internalization of the E12 phage antibody, scFv and immunoliposomes into CHO/EGFR and CHO cells. The E12 phage antibody was detected with α -M13-biotin followed by streptavidin–phycoerythrin. The E12 scFv was directly labeled with FITC and immunoliposomes containing the fluorescent dye HPTS constructed. Binding and internalization into CHO/EGFR and CHO cells of the phage antibodies, scFv and immunoliposomes was analyzed by confocal microscopy on either fixed cells after stripping the cell surface of antibody (for phage antibodies) or on live cells with no stripping of the cell surface (for scFv and immunoliposomes). Phage antibodies, scFv, and immunoliposomes showed intracellular staining. Where the antibody was not removed from the cell surface (scFv and immunoliposomes) surface staining was also observed.

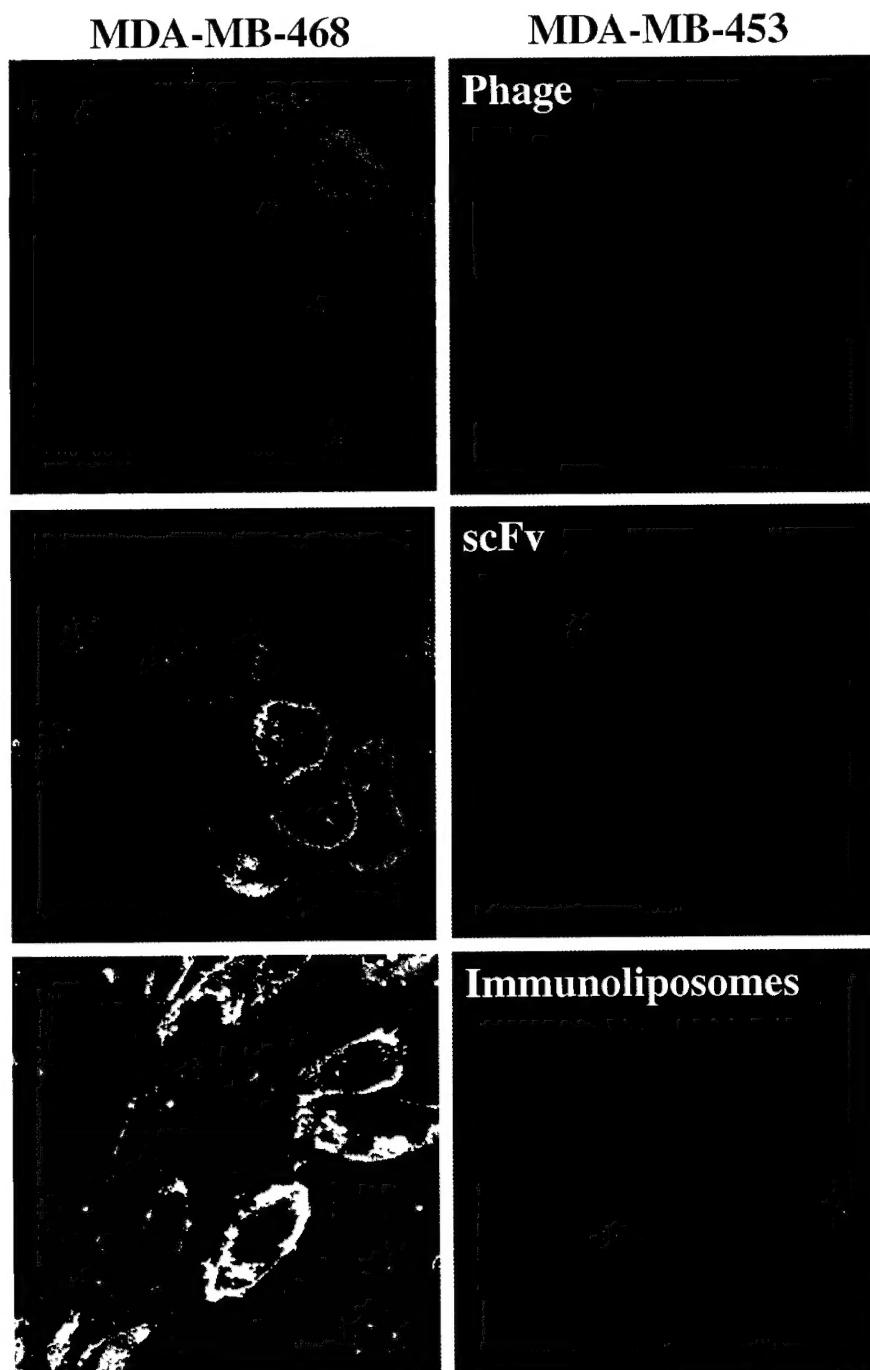


Fig. 6. Binding and internalization of the E12 phage antibody, scFv and immunoliposomes into EGFR expressing MDA-MB-468 and EGFR negative MDA-MB-453 cells. The E12 phage antibody was detected with α -M13-biotin followed by streptavidin-phycoerythrin. The E12 scFv was directly labeled with FITC and immunoliposomes containing the fluorescent dye HPTS constructed. Binding and internalization into EGFR expressing MDA-MB-468 and EGFR negative MDA-MB-453 cells of the phage antibodies, scFv and immunoliposomes was analyzed by confocal microscopy on either fixed cells after stripping the cell surface of antibody (for phage antibodies) or on live cells with no stripping of the cell surface (for scFv and immunoliposomes). Phage antibodies, scFv and immunoliposomes showed intracellular staining. Where the antibody was not removed from the cell surface (scFv and immunoliposomes) surface staining was also observed.

the need to express and purify the target antigen in order to select antibodies. This could significantly speed development of antibodies to genes discovered as part of genomic sequences.

Selection on EGFR overexpressing A431 cells resulted in more efficient selection of EGFR antibodies than selection on CHO/EGFR cells (a higher percentage of antigen binding clones, although both selections yielded two unique antibodies). This occurred despite depletion of non-EGFR binding phage using the parental CHO cell line. In fact, the depletion was found to have been insufficient as FACS analysis showed that polyclonal phage bound both CHO/EGFR cells and CHO cells. The difference in efficiency between the two selections could potentially be attributed to a greater cell surface receptor density on A431 cells than on CHO/EGFR cells. Although not quantified, a Western blot of the cell lysates demonstrated a greater signal for A431 cells as compared to CHO/EGFR cells. Interestingly, one antibody was common to both selections (A431 or CHO/EGFR), whereas each of the remaining two antibodies were only selected on one of the cell types (A431 or CHO/EGFR). This result suggests that selection on multiple cell types may yield a greater number of antibodies.

The phage antibodies generated in this and previous work (Poul et al., 2000) were internalized by cells as determined by immunofluorescence and confocal microscopy. In both reports, the phage antibodies were selected from libraries where monomeric scFv were displayed as single copies in a phagemid system. In fact, all large non-immune libraries display monovalent antibody fragments (either scFv or Fab) as single copies using a phagemid vector. Since antibodies typically need to be bivalent to crosslink receptors and trigger endocytosis (Heldin, 1995; Yarden, 1990), successful selection of internalizing antibodies from phagemid libraries would require that: (1) the scFv formed spontaneous scFv dimers (diabodies) on the phage surface, as has been reported for some scFvs; (2) the monovalent scFv mimicked the natural receptor ligand leading to receptor aggregation and endocytosis or (3) increased phage display levels led to greater than one scFv per phage. In our previous work, the two scFvs studied extensively (anti-ErbB2 and anti-transferrin receptor) were stable scFv mono-

mers in solution and were significantly endocytosed into cells as monomeric scFv. In the case of the transferrin receptor antibody, the scFv was a ligand mimetic and could compete with the natural ligand transferrin for binding to the receptor. In the case of the ErbB2 scFv, the mechanism by which it was endocytosed as a monomer is unknown. In the present work, the E12 scFv shows evidence of spontaneous dimerization (diabody formation) by gel filtration which could explain how it could crosslink receptors and trigger endocytosis. Interestingly, the purified scFv monomer (separated from dimer) shows significantly more surface membrane staining than intracellular staining (Figs. 5 and 6), especially compared to the multimeric immunoliposomes or to phage (which could be displaying dimeric scFv). In the case of the other two EGFR scFvs (which form stable monomers) the mechanism of endocytosis is unclear. We did not study whether the EGFR antibodies were ligand mimetics.

The approach described would be limited to those receptors capable of undergoing endocytosis. While this eliminates some useful cell surface targets, ligand binding and receptor internalization is a common mechanism for receptor and signaling regulation. Since most antibodies need to be bivalent to crosslink receptors and be efficiently endocytosed, one mechanism to increase the applicability of this selection methodology would be to construct bivalent diabody libraries in a phagemid vector or scFv libraries in a multivalent phage vector. This should open up the selection approach to more epitopes on more target antigens. Our model system results indicate that the most efficient selection format would be display on phage (Becerril et al., 1999), an approach which is presently under investigation.

The therapeutic utility of scFvs generated by this approach depends on the specific molecules to be targeted by the antibodies and the properties of the antibody. For many therapeutic approaches (immunotoxins, immunoliposomes, gene therapy) intracellular delivery of the toxic molecule is essential. Other approaches, for example bispecific antibodies or enzyme activated prodrugs, require that the antibody and effector molecule remain on the cell surface. Based on the present results (and our prior publication), the selection strategy described generates two types of scFv: those that are endocytosed in

their monomeric form (probably the majority of scFvs) and those that remain on the cell surface as monomers but are endocytosed when dimeric or multimeric. scFvs which are endocytosed as monomers could only be used for targeting effector molecules that are active intracellularly. scFvs which are primarily endocytosed as dimers could be used to leave effector molecules on the cell surface (when used as monomeric antibody fragments) or to deliver drugs intracellularly (when used as bivalent diabodies or IgG or when targeting multivalent nanoparticles).

In conclusion, we report the successful selection of EGFR antibodies from a phage library by selection for internalization into overexpressing cells or transfected cells. The scFvs are specific for EGFR expressing cells and can be used to target nanoparticles for intracellular drug delivery. Use of a transfected cell line allows selection of antibodies to native receptors without the need for protein expression and purification, significantly speeding the generation of targeting antibodies to genomic sequences.

Acknowledgements

CHO/EGFR cells were designed by Dr. Peter Morrison. We thank Will Tseng for his assistance with protein expression and purification. This work was partially supported by DAMD-17-98-1-8189, DAMD-17-97-1-7250, and NIH/NCI 5 P50 CA 58207 and by NIH grant RO1 AR40352-03. We acknowledge C.M. for her inspiration.

References

- Andersen, P., Stryhn, A., Hansen, B., Fugger, L., Engberg, J., Buus, S., 1996. A recombinant antibody with the antigen-specific, major histocompatibility complex-restricted specificity of T cells. *Proc. Natl. Acad. Sci. USA* 93, 1820.
- Baselga, J., Mendelsohn, J., 1994. The epidermal growth factor receptor as a target for therapy in breast carcinoma. *Breast Cancer Res. Treat.* 29, 127.
- Becerril, B., Poul, M., Marks, J.D., 1999. Toward selection of internalizing antibodies from phage libraries. *Biochem. Biophys. Res. Commun.* 255, 386.
- Benedict, C.A., MacKrell, A.J., Anderson, W.F., 1997. Determination of the binding affinity of an anti-CD34 single-chain antibody using a novel, flow cytometry based assay. *J. Immunol. Methods* 201, 223.
- Breitling, S.D., Sechhaus, T., Klewinghaus, I., Little, M., 1991. A surface expression vector for antibody screening. *Gene* 104, 147.
- Cai, X., Garen, A., 1995. Anti-melanoma antibodies from melanoma patients immunized with genetically modified autologous tumor cells: selection of specific antibodies from single-chain Fv fusion phage libraries. *Proc. Natl. Acad. Sci. USA* 92, 6537.
- Carter, P., Presta, L., Gorman, C.M., Ridgway, J.B., Henner, D., Wong, W.L., Rowland, A.M., Kotts, C., Carver, M.E., Shepard, H.M., 1992. Humanization of an anti-p185HER2 antibody for human cancer therapy. *Proc. Natl. Acad. Sci. USA* 89, 4285.
- Chrysogelos, S.A., Dickson, R.B., 1994. EGF receptor expression, regulation, and function in breast cancer. *Breast Cancer Res. Treat.* 29, 29.
- De Bellis, D., Schwartz, I., 1990. Regulated expression of foreign genes fused to lac: control by glucose levels in growth medium. *Nucleic Acids Res.* 18, 1311.
- De Jong, J.S., van Diest, P.J., van der Valk, P., Baak, J.P.A., 1998. Expression of growth factors, growth inhibition factors, and their receptors in invasive breast cancer. II Correlations with proliferation and angiogenesis. *J. Pathol.* 184, 53.
- de Kruijff, J., Terstappen, L., Boel, E., Logtenberg, T., 1995. Rapid selection of cell subpopulation-specific human monoclonal antibodies from a synthetic phage antibody library. *Proc. Natl. Acad. Sci. USA* 92, 3938.
- Einfeld, D.A., Brown, J.P., Valentine, M.A., Clark, E.A., Ledbetter, J.A., 1988. Molecular cloning of the human B cell CD20 receptor predicts a hydrophobic protein with multiple transmembrane domains. *EMBO J.* 7, 711.
- Garcia de Palazzo, I.E., Adams, G.P., Sundareshan, P., Wong, A.J., Testa, J.R., Bigner, D.D., Weiner, L.M., 1993. Expression of mutated epidermal growth factor receptor by non-small cell lung carcinomas. *Cancer Res.* 53, 3217.
- Ghete, M.A., Podar, E.M., Ilgen, A., Gordon, B.E., Uhr, J.W., Vitetta, E.S., 1997. Homodimerization of tumor-reactive monoclonal antibodies markedly increases their ability to induce growth arrest or apoptosis of tumor cells. *Proc. Natl. Acad. Sci. USA* 94, 7509.
- Harris, A.L., 1994. What is the biological, prognostic, and therapeutic role of the EGF receptor in human breast cancer? *Breast Cancer Res. Treat.* 29, 1.
- Heldin, C.-H., 1995. Dimerization of cell surface receptors in signal transduction. *Cell* 80, 213.
- Hochuli, E., 1988. Large-scale chromatography of recombinant proteins. *J. Chromatogr.* 444, 293.
- LeMaistre, C.F., Meneghetti, C., Howes, L., Osborne, C.K., 1994. Targeting EGF receptor in breast cancer. *Breast Cancer Res. Treat.* 32, 97.
- Marks, C., Marks, J.D., 1996. Phage libraries – a new route to clinically useful antibodies. *New Engl. J. Med.* 335, 730.
- Marks, J., Hoogenboom, H., Bonnert, T., McCafferty, J., Griffiths, A., Winter, G., 1991. By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *J. Mol. Biol.* 222, 581.

- Marks, J.D., Ouwehand, W.H., Bye, J.N., Finnern, R., Gorick, B.D., Voak, D., Thorpe, S., Hughes-Jones, N.C., Winter, G., 1993. Human antibody fragments specific for human blood group antigens from a phage display library. *Biotechnology* 11, 1145.
- Morrison, P., Takishima, K., Rosner, M.R., 1993. Role of threonine residues in regulation of the epidermal growth factor receptor by protein kinase C and mitogen-activated protein kinase C and mitogen-activated protein kinase. *J. Biol. Chem.* 268, 15536.
- Odaka, M., Kohda, D., Lax, I., Schessinger, J., Inagaki, F., 1997. Ligand-binding enhances the affinity of dimerization of the extracellular domain of the epidermal growth factor receptor. *J. Biochem.* 122, 116.
- Park, J.W., Kirpotin, D., Hong, K., Colbern, G., Shalaby, R., Shao, Y., Meyer, O., Nielsen, U., Marks, J., Benz, C.C., Papahadjopoulos, D., 1998. Anti-HER2 immunoliposomes for targeted drug delivery. *Med. Chem. Res.* 8, 383.
- Parren, P., Fiscaro, P., Labrijn, A., Binley, J., Yang, W., Ditzel, H., Barbas, C.R., Burton, D., 1996. In vitro antigen challenge of human antibody libraries for vaccine evaluation: the human immunodeficiency virus type 1 envelope. *J. Virol.* 70, 9046.
- Pereira, S., Maruyama, H., Siegel, D., Van Belle, P., Elder, D., Curtis, P., Herlyn, D., 1997. A model system for the detection and isolation of a tumor cell surface antigen using antibody phage display. *J. Immunol. Methods* 203, 11.
- Poul, M., Becerril, B., Nielsen, U., Morisson, P., Marks, J.D., 2000. Selection of tumor specific internalizing human antibodies from phage libraries. *J. Mol. Biol.* 301, 1149.
- Sanna, P., Williamson, R., De Logu, A., Bloom, F., Burton, D., 1995. Directed selection of recombinant human monoclonal antibodies to herpes simplex virus glycoproteins from phage display libraries. *Proc. Natl. Acad. Sci. USA* 92, 6439.
- Sawyer, C., Embleton, J., Dean, C., 1997. Methodology for selection of human antibodies to membrane proteins from a phage-display library. *J. Immunol. Methods* 204, 193.
- Schier, R., Marks, J.D., Wolf, E.J., Apell, G., Wong, C., McCartney, J.E., Bookman, M., Huston, J., Houston, L.L., Weiner, L.M., Adams, G.P., 1995. In vitro and in vivo characterization of a human anti-c-erbB-2 single-chain Fv isolated from a filamentous phage antibody library. *Immunotechnology* 1, 73.
- Schier, R., McCall, A., Adams, G.P., Marshall, K.W., Merritt, H., Yim, M., Crawford, R.S., Weiner, L.M., Marks, C., Marks, J.D., 1996. Isolation of picomolar affinity anti-c-erbB2 single-chain Fv by molecular evolution of complementary determining regions in the center of the antibody binding site. *J. Mol. Biol.* 263, 551.
- Sheets, M.D., Amersdorfer, P., Finnern, R., Sargent, P., Lindqvist, E., Schier, R., Hemingsen, G., Wong, C., Gerhart, J.C., Marks, J.D., 1998. Efficient construction of a large non-immune phage antibody library: the production of high-affinity human single-chain antibodies to protein antigens. *Proc. Natl. Acad. Sci.* 95, 6157.
- Siegel, D., Chang, T., Russell, S., Bunya, V., 1997. Isolation of cell surface-specific human monoclonal antibodies using phage display and magnetically-activated cell sorting: applications in immunohematology. *J. Immunol. Methods* 206, 73.
- Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A. et al., 1989. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 244, 707.
- Taji, H., Kagami, Y., Okada, Y., Andou, M., Nishi, Y., Saito, H., Seto, M., Morishima, Y., 1998. Growth inhibition of CD20-positive B lymphoma cell lines by IDEC-C2B8 anti-CD20 monoclonal antibody. *Jpn. J. Cancer Res.* 89, 748.
- Tzahar, E., Pinkas-Kramarski, R., Moyer, J.D., Klapper, L.N., Alroy, I., Levkowitz, G., Shelly, M., Henis, S., Eisenstein, M., Ratzkin, B.J., Sela, M., Andrews, G.C., Yarden, Y., 1997. Bivalence of EGF-like ligands drives the erbB signalling network. *EMBO J.* 16, 4938.
- Van Ewijk, W., de Kruif, J., Germeraad, W., Berendes, P., Ropke, C., Platenburg, P., Logtenberg, T., 1997. Subtractive isolation of phage-displayed single-chain antibodies to thymic stromal cells by using intact thymic fragments. *Proc. Natl. Acad. Sci. USA* 94, 3903.
- Vieira, A.V., Lamaze, C., Schmid, S.L., 1996. Control of EGF receptor signalling by clathrin-mediated endocytosis. *Science* 274, 2086.
- Watters, J.M., Telleman, P., Junghans, R.P., 1997. An optimized method for cell-based phage display panning. *Immunotechnology* 3, 21.
- Yarden, Y., 1990. Agonistic antibodies stimulate the kinase encoded by the neu protooncogene in living cells but the oncogenic mutant is constitutively active. *Proc. Natl. Acad. Sci. USA* 87, 2569.